Original Article

Bcl-2 in suppressing neuronal apoptosis after spinal cord injury

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BACKGROUND: Apoptosis plays an important role in central neural diseases and trauma. B-cell lymphoma/Leukemia-2 (Bcl-2) can inhibit apoptosis in a wide variety of cells including neurons. In this experiment, by studying Bcl-2 over-expression transgenic (TG) mice subjected to spinal cord injury (SCI), we investigated whether Bcl-2 could reduce posttraumatic neuronal apoptosis, reduce the range of damage, and improve the behavioral functional recovery after contusive SCI.

METHODS: Nine Bcl-2 TG mice and nine control mice were subjected to SCI of moderate severity at T10, with the use of weight dropping (WD) method (impact force 2.5×3.0 g/cm). At times up to 1 day, 7 days and 14 days after SCI, functional deficits were evaluated with Basso, Beattie, and Bresnahan (BBB) scales, and apoptosis of neurons was investigated by using the TUNEL method. Another three control mice only underwent lamina opening, but were not subjected to SCI, to provide blank comparison.

RESULTS: The mean functional scores for the control mice (5.05 ±0.35) were lower than those for the Bcl-2 TG mice (5.45 ±0.15), although the unpaired T-test revealed no significant difference (P=0.67). On the other hand, the number of TUNEL positive neurons and integrated option density (IOD) scores for the Bcl-2 TG mice were both significantly lower than those for the control mice (P<0.05).

CONCLUSIONS: This experiment suggests that overexpression of Bcl-2 may suppress neuronal apoptosis after SCI. Bcl-2 may be an important factor within the central nervous system that can relieve the damage after trauma.

KEY WORDS: Spinal cord injury; Bcl-2; Apoptosis of neurons; Weight dropping; Transgenic mouse

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INTRODUCTION

Spinal cord injury (SCI) is commonly associated with a high disability, and it often occurs in young people under the age of 30 years. It is reported that the average incidence of SCI is 15-40/million in developed countries.[1] An average of over $750 000 will be spent on each paralyzed patient after SCI, and merely in the USA over $600 million is spent every year in this way. In China, it is estimated that there are more than 1 million paralytic patients after SCI. Unfortunately, as the recovery mechanism of central nerve damage is yet far from clear, the curative effect is often poor.

The pathophysiological processes of SCI are complex. There are two main forms of neuron damage in SCI: primary injury and secondary injury. Primary injury refers to the condition that the violence destroys the cellular structure of neurons directly and causes irreversible metabolic disorder, which leads to the immediate death of the cells.[2,3] Then the secondary injury follows, caused by cellular and molecular processes that involve apoptosis and other mechanisms.[4,5]

It has been proved that B-cell lymphoma/leukemia-2 (Bcl-2) can suppress apoptosis in a variety of cell systems, including neurons, either by preventing the release of

cytochrome c from mitochondria or by binding to the apoptosis-activating factor (APAF-1). Several studies have demonstrated that over-expression of Bcl-2 prevents neurons from cell death induced by free radicals,[6] hypoxia,[7] or growth factor deprivation.[8]

However, we have not yet found systemic studies on the effect of Bcl-2 on suppressing neuronal apoptosis *in vivo* after SCI. In this experiment, by studying Bcl-2 over-expression transgenic (TG) mice subjected to SCI, we investigated whether Bcl-2 could reduce posttraumatic neuronal apoptosis, reduce the damage range, and improve the behavioral functional recovery after contusive SCI.

METHODS

Construction of human Bcl-2 expression plasmid

We obtained human Bcl-2 mRNA sequence (code: BC027258) from National Center for Biotechnology Information (NCBI). According to this template, we used standard RT-PCR kit for reverse transcription, and after PCR cloned the DNA sequence into BAP-2 plasmid. Through DNA sequencing, we confirmed that the direction and sequence of the inserted fragments were correct, thus completing the construction of Bcl-2 expression plasmid.

Generation of human Bcl-2 transgenic mice

The TG mice were generated in Shanghai Research Center for Model Organisms by the routine microinjection of linearized DNA of the Bcl-2 gene into fertilized eggs of mice. These eggs were then transferred into pseudopregnant mice. Both the donor and recipient mice were F1 hybrid mice (DBA×C57BL/6J). After that the mice were raised in the SPF-class animal room.

Identification of human Bcl-2 transgenic mice

The TG mice and control mice (of the same strain) at the age of 2 weeks were genotyped by polymerase chain reaction (PCR) amplification of genomic DNA extracted from the tail. The PCR procedure involved an initial denaturation step for 4 minutes at 95 °C, followed by 34 cycles of denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 64 °C, and extension for 30 seconds at 72 °C. A final extension was performed for 10 minutes at 72 °C. The PCR primers we used were 5'-TGAACAAGCAGCCTCCTTCC-3' (forward), and 5'-CCTGTCCTTGTCACCCTTTC -3' (reverse). Western blotting analysis was performed for the Bcl-2 TG and control mice.

Experimental groups

All mice used in this experiment were mature males aged 8 weeks and weighing 22.3±2.4 g. There were nine human Bcl-2 TG mice, and 12 littermates as control mice. The TG mice were backcrossed for six generations with C57BL/6J background mice. Nine Bcl-2 TG mice and nine control mice were subjected to SCI of moderate severity at T10, using the weight dropping (WD) method (SCI group). Three control mice only underwent lamina opening, but were not subjected to SCI, to provide blank comparison (sham group).

Establishment of SCI models

All mice in the SCI group were subjected to controlled-impact SCI of moderate severity produced with a spine impact device (Fudan University, Shanghai, China) using the weight dropping method. Dark/light cycles of 12 hours were maintained, and food and water were available. The mice were anesthetized and maintained with chloral hydrate (10%, 5 mL/kg). During surgery, the rectal temperature was monitored and maintained at 34.5 to 36.0 °C with a heating pad. After a deep level of anesthesia had been established, the surgical area was cleaned with iodinated solution. A midline incision was made in the skin from the T9 level to the T13 level. The muscles of the vertebral column at T10 were incised on both sides to expose the spinous processes. A T10 laminectomy was performed, leaving the dura mater intact. The animals were then placed in a prone position. All mice received moderate SCI, with an impact strength of 2.5×3.0 g/cm. After the impact, the skin was sutured. After operation all mice were raised in the SPF-class animal room. Bladder expression was performed twice per day until reflex bladder emptying was established. All surgical procedures, interventions, and preoperative and postoperative care were performed by specialists in Fudan University School of Medicine.

Functional assessments

Functional recovery of the mice subjected to SCI were evaluated by Basso, Beattie, and Bresnahan (BBB) scales which is a method for assessing hind limb and coordinated motor functions in mice with spine injury.[9,10] All animals in the SCI group were examined by using behavioral function tests to establish baseline scores before surgery. At times up to 1 day, 7 days and 14 days after SCI, BBB scales of hind limb movements were observed for examination of functional deficits and recovery of the control and TG mice. Both hind limbs were examined for

each mouse, in a blinded manner.

HE staining

At each given time node (for the SCI group: 1 day, 7 days, 14 days; for the sham group: 14 days), after observation, the animals were killed and their spinal cords, including the T10 level, were processed for HE staining to examine the difference in lesion between the control and TG mice. The mice were deeply anesthetized and were transcardially perfused with 20 mL of saline solution, followed by 60 mL of 4% paraformaldehyde. The spinal cords were embedded in paraffin, sectioned in the sagittal plane (5-μm-thick sections), and stained with hematoxylin and eosin. The regions of spinal cord anterior horn were reviewed under an Olympus photomicroscope (magnification×400).

TUNEL staining

Apoptotic neurons were identified using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method.[11] The assay was performed using TUNEL kit from Roche Corp (Germany). Deparaffinized and rehydrated specimens were incubated in proteinase K (40 μg/mL) for one hour at 37 °C, and were then treated with 3% H_2O_2 in methanol for 30 minutes at room temperature. After adding equilibration buffer for five minutes at room temperature, the terminal deoxynucleotidyl transferase enzyme was pipetted to the sections and incubated at 37 °C for two hours. After the reaction was stopped by stop buffer, anti-digoxigenin peroxidase was added to the slides, followed by incubation for 30 minutes at 37 °C. Slides were stained with diaminobenzine for 10 minutes and counterstained with hematoxylin. Review and photography of all histologic preparations were carried out with an Olympus photomicroscope.

TUNEL-positive cell counts on the spinal cord anterior horn were calculated under magnification (×400).

For semiquantitative evaluation of TUNEL image in the spine, the specimens were assessed by integrated option density (IOD) score. Formula: IOD = area density × average optical density.

Statistical analysis

Final outcomes such as behavioral scores, the number of TUNEL positive cells, and IOD scores for all mice were expressed as mean±SD. The results were analyzed with the unpaired *t* test. *P* values<0.05 were considered statistically significant.

RESULTS

Establishment of Bcl-2 overexpression transgenic mice

Expression plasmid of human Bcl-2 was confirmed correct by restriction enzyme digestion and sequencing.

In total, 415 fertilized eggs were microinjected with human Bcl-2 DNA. After these eggs were transferred into 16 pseudopregnant mice, 57 mice were born and genotyped. Of these mice, 5 (#5, #6, #14, #26, #54) were positive. They were called founder mice. Lineage #6, #14 mice were established successfully by backcrossing positive founder mice with wild-type C57BL/6J mice. Further characterization of lineage #6, #14 mice was done using PCR and Western blotting. PCR analysis of genomic DNA from TG mice showed a 457-bp band, indicating that the Bcl-2 genome was integrated into the mouse chromosome (Figure 1). Western blotting analysis of spleen and spine homogenates showed over-expression of Bcl-2 protein in the TG mice (Figure 2).

Postoperative conditions

There were no deaths during the experiment. And no evidence of pneumonia or bladder infection was noted among all animals. No animal received antibiotics.

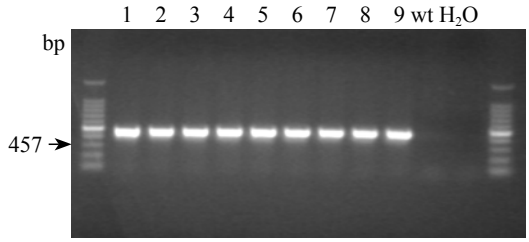

Figure 1. PCR analysis of the Bcl-2 TG mice and one of the control mice (wt). The Bcl-2 band can be observed at 457 base pairs (bp) for all Bcl-2 TG mice (lanes 1–9).

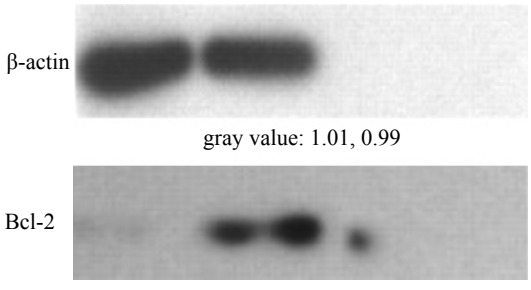

Figure 2. Western blotting analysis of Bcl-2 in the spinal cord of TG mice. From left to right were negative specimens and positive specimens. Lane left represented wild-type mouse, and lane right represented Bcl-2 TG mouse. Top signals showed β-actin, and bottom signals indicated Bcl-2.

Functional recovery

After surgery all animals in the SCI group were paralyzed in hinder limbs. In the latter days gradual improvement in hind limb function was observed in both TG and control mice, but complete recovery during the 14 days was not observed. The mean scores for the control mice in these behavioral tests were lower than those for the Bcl-2 TG mice, although the unpaired t test revealed no significant differences (Table 1, Figure 3).

Though there was no marked difference between the two groups, mice in the control group had worse functional recovery than the TG mice 14 days after injury.

HE staining

Hematoxylin and eosin staining revealed typical neuron damage in all SCI mice, which was absent in the sham group. Within the first day after SCI, we noted that neurons in most cases were swelling and a few characterized with dispersed chromatin. Seven days later, many of the neurons were ballooned. Ballooned neurons(BNs) displayed several morphological changes such as swelling, chromatolysis, and disintegration of the nuclear and cytoplasmic membrane. Among BNs, a smaller number of neurons with nuclear and cytoplasmic condensation as well as clumping of chromatin, was observed. Groups of microglial cells were accumulated, although the grade in the TG mice was slighter than that in the controls. Fourteen days later, neuron loss was obvious in each specimen investigated. The specimens of the spinal cord from the TG mice showed a smaller region of damage compared with that of the control mice. Neuron loss at this point in the TG mice was less than that in their littermates (Figure 4).

TUNEL staining

TUNEL staining revealed typical neuron apoptosis in all SCI mice, which was almost absent in the sham group. TUNEL positive neurons were characterized by brown

Table 1. BBB scores in the TG mice and control mice at different time points (n=3, mean ± SD)

Groups	Time point (day)		
	1	7	14
TG	0.33 ± 0.25	3.33 ± 1.52	5.00±2.00
Control	0.30 ± 0.10	3.00 ± 1.73	4.33±1.52
P value	0.842	0.815	0.67

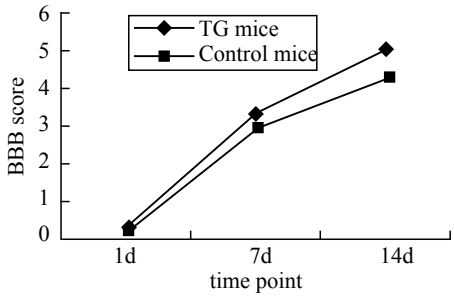

Figure 3. BBB score variation at different time points with mice undergoing SCI

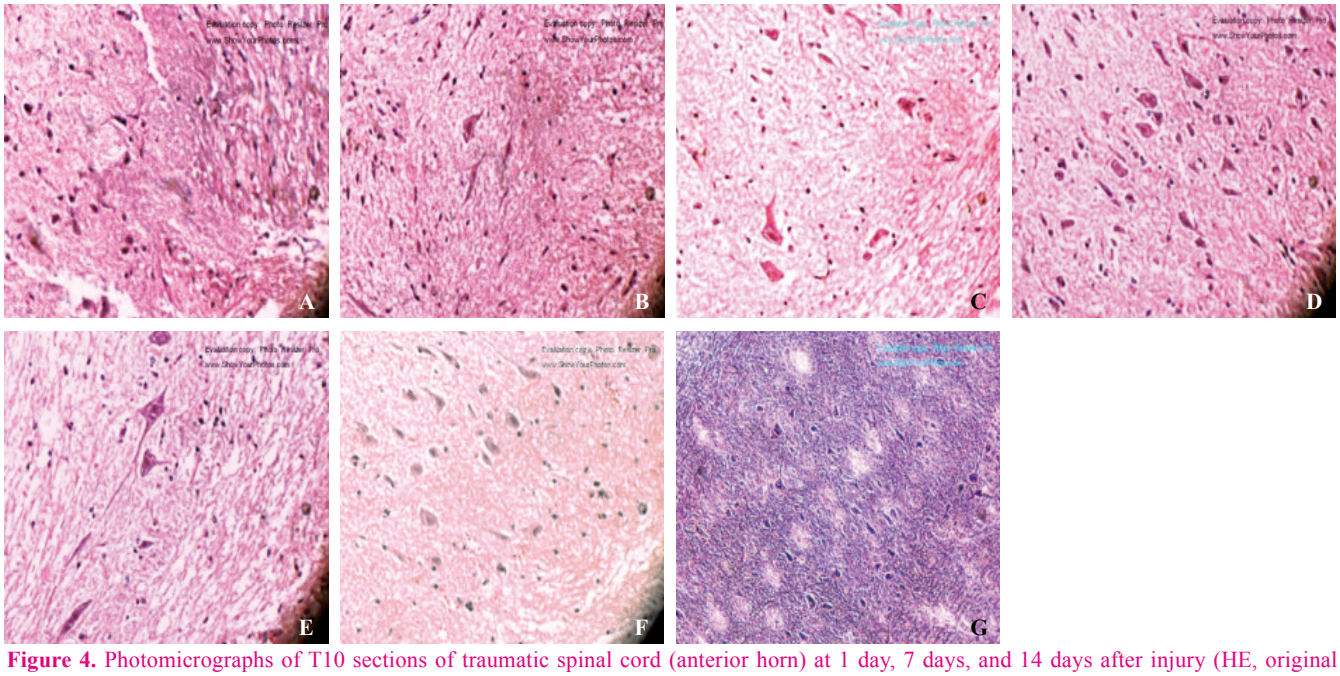

Figure 4. Photomicrographs of T10 sections of traumatic spinal cord (anterior horn) at 1 day, 7 days, and 14 days after injury (HE, original magnification×400). **A, C, E**: TG mice; **B, D, F**: control mice. The arrows showed the damaged neurons, and the scale was 100 μm.

granules in the nucleus, while the nucleus of TUNEL negative neurons counterstained with hematoxylin showed light blue. Within the first day after SCI, we observed TUNEL positive neurons in the TG mice and control mice. Then these positive neurons accumulated gradually, and reached the maximum 7 days later. After that, TUNEL positive neurons decreased stably. But we could still find them on the specimens 14 days after SCI. The number of TUNEL positive neurons from the TG mice was markedly lower than that from the control mice. Semiquantitative analysis of the specimens of the spinal cord, expressed as a mean IOD score, showed a significant reduction of neuronal apoptosis in the TG mice compared with that in their littermates (Figure 5, Tables 2 and 3).

Table 2. The number of TUNEL positive neurons in the TG mice and control mice at different time points (n=3, mean ± SD)

Groups	Time point (day)		
	1	7	14
TG	4.33±1.52	5.30±1.52	3.67±2.08
Control	11.66±5.03	13.67±4.50	12.00±4.00
Sham			0.33±0.577
P value	0.042	0.039	0.033*, 0.046#, 0.007Δ

*: TG mice vs. control mice; #: TG mice vs. sham group; Δ: Control mice vs. sham group

Table 3. IOD score in the TG mice and control mice at different time points (n=3, mean ±SD)

Groups	Time point (day)		
	1	7	14
TG	291.67±97.6	742±33	458.33±112.7
Control	1860±110.75	4105±33	2436.33±228.01
P value	0.000	0.000	0.000

DISCUSSION

As mentioned previously, primary injury can lead to secondary injury which often involves apoptosis.[12,13] Apoptosis is an active gene-directed cell death process that plays an important role in the development of many CNS diseases.[14]

In contrast to the sham group in our study, which was virtually free from any TUNEL positive cells, the SCI group showed the evidence of neuronal apoptosis at the injury site. The TUNEL positive neurons showed morphological features of apoptosis: condensation of the nucleus and cytoplasm, margination of the condensed chromatin, blebbing of the nucleus and plasma membrane, and segregation and disintegration of the nucleus. These changes appeared early one day after spine cord injury. The number of apoptotic neurons reached the maximum 7 days after injury and then decreased. Fourteen days after injury a significant neuron loss was observed in the specimens. Some reports indicated that similar changes after SCI were noted in other animal models including rabbits. Thus our study indicated that apoptosis of neurons may be responsible

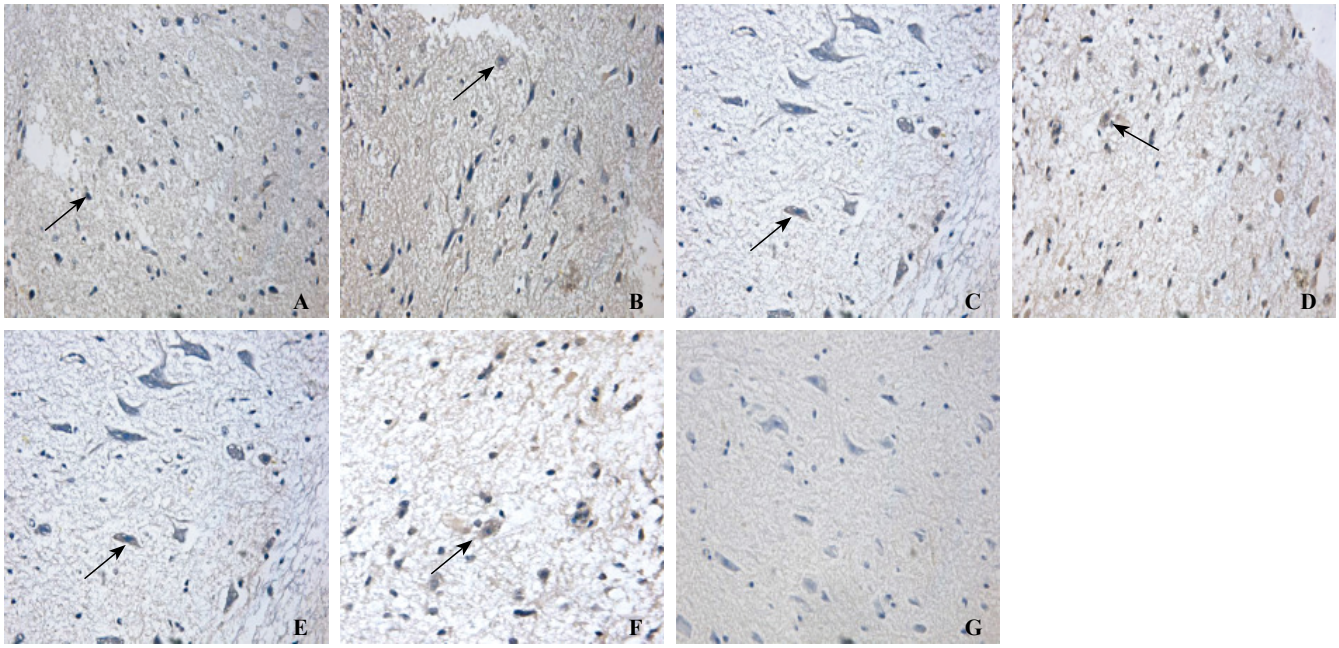

Figure 5. TUNEL images of T10 sections of traumatic spinal cord (anterior horn) at 1 day, 7 days, 14 days after injury (TUNEL staining×400). **A, C, E**: TG mice; **B, D, F**: control mice; G: sham mice.The arrows showed the positive neurons, and the scale was 100 μm.

for secondary injury after traumatic SCI. If apoptosis of neurons after injury can be inhibited effectively, the loss of neural tissue could be reduced significantly, and the prognosis of SCI may be improved.

Furthermore, in our study the TUNEL positive neurons in the TG mice after injury were significantly less than those in the control mice. Such phenomenon may be explained by the anti-apoptosis activity of Bcl-2. Though we did not observe significant functional recovery assessed with BBB scores, the mean score was generally higher for the Bcl-2 TG mice than that for the control mice. In a follow-up period, Bcl-2 over-expression may have beneficial effects on spinal function in later stages, and an obvious recovery may be seen.

The commitment step of apoptosis is regulated by the expression of genes of the Bcl-2 protein family, which includes both proapoptotic and antiapoptotic members.[15] Most of them, including Bcl-2 protein, are antiapoptotic members. Recent studies have demonstrated that Bcl-2 protein can block the release of cytochrome c from the mitochondria into the cytosol, thus preventing the upstream activation of several caspases, including caspase-3. [16-18] Caspase-3 is known as the vital enzyme involved in apoptosis. Bcl-2 has shown an antiapoptotic function in many types of cells, including neurons. One study indicated that over-expression of Bcl-2 by gene transfer or in transgenic mice reduced infarction after permanent and transient focal ischemia. Expression of the apoptosis-inhibitory Bcl-2 gene under the control of tissue specific promoters has been proved to be capable of protecting various types of cells from apoptosis, particularly motor neurons of the spinal cord.[19]

After injury, Bcl-2 could be induced in surviving neurons.[20] This suggests that Bcl-2 may be a protective factor within the CNS that may relieve neuropathological sequelae after central nerve injury such as SCI. The molecular mechanism of the protective function by overexpression of Bcl-2 may be explained as follows: First, the antiapoptotic action of Bcl-2 may be the main action. This has been reported in several cases, and has been demonstrated through our research. In addition to the antiapoptotic action, Bcl-2 has been recently proved to act as an antioxidant.[21] After SCI, ischemia reperfusion and inflammation produce oxygen free radicals in the spine. It has been proved that these free radicals can increase damage in the spine. Overexpression of Bcl-2 in cultured neurons has been protective against various noxious stimuli inducing free radicals.[22] In our study, we observed that the proliferation of microglial cells which indicate inflammation within the CNS was slighter in the TG mice. Hence we suppose that the antioxidant action of Bcl-2 might contribute in part to the neuronal protection.

Both induction of the Bcl-2 oncogene with a recombinant adenovirus vector and direct injection into the spinal cord of a deoxyribonucleic acid plasmid encoding the human Bcl-2 gene could reduce delayed neuronal loss or the zone of microscopic injury to the contused rat spinal cord.[23-25] Our study demonstrated neuroprotective effects of Bcl-2 expression in a mouse model of SCI. Thus gene therapy with Bcl-2 gene induction in acute SCI may be a potentially effective method to relieve neuropathological sequelae.

Our study demonstrated a significant reduction in posttraumatic tissue loss and neuronal apoptosis in the spinal cord of the Bcl-2 TG mice after SCI. The results suggest that over-expression of Bcl-2 may play a protective role in the recovery after SCI by suppressing neuronal apoptosis. Bcl-2 may be an important factor within the CNS that can relieve the damage after trauma. Further studies are needed to explore the possibility of the gene therapy using the Bcl-2 oncogene in acute SCI.

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